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JOURNAL ARTICLE

Evaluation of Trace-Element Interactions Using Cultured Alveolar Macrophages

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ABSTRACT

It is important to consider the interactions of toxic trace elements in an evaluation of the toxicity of environmental pollutants. The in vitro toxicity screening system developed at the Environmental Research Center using the rabbit alveolar macrophage provides a particularly useful system for evaluating trace-element interactions since crude particulates containing multiple trace elements can be introduced into the system.

Mercury, cadmium, and vanadium are the three most toxic metals that have been tested in the macrophage system. When cells were exposed simultaneously to toxic concentrations of cadmium or mercury and nontoxic concentrations of sodium selenite, the macrophages had significantly higher viability, cell number, and adenosine triphosphate concentration than when they were exposed to the cadmium or mercury salts alone. In analogous experiments, zinc was found to protect against cadmium toxicity, and copper protected the macrophage against the lytic effects of vanadium.

The phagocytic rabbit alveolar macrophage is a particularly appropriate cell in which to evaluate the cellular toxicity of airborne environmental chemicals, including particulates. Previous studies from the Environmental Research Center[1,2] have reported the use of this cell type in a screening system to rank the cytotoxicity of a series of metallic chlorides.

A modification of this system has been used recently to compare the relative cytotoxicity of a series of size fractionated industrial particulates.[3] Spark-source mass spectrometry was used to determine the elemental concentration of approximately 70 elements in these

particulate samples. The toxicity of the particulates was then predicted on the basis of a mathematical summation of the toxicities of the individual elements present in the sample.[4] This method does not take into account interactions that may occur between elements. The predicted toxicity of one-third of these samples was nearly twice the toxicity that was actually observed when the particulates were tested with the screening system that uses the rabbit alveolar macrophage. This suggests that the cytotoxic response of the macrophages may reflect antagonistic trace-element interactions resulting from a combination of elements in the sample.

The trace-element interactions reported in whole animals are antagonistic rather than synergistic[5] and include interactions between: cadmium and zinc, cadmium and copper, cadmium and selenium, copper and zinc, mercury and selenium, lead and calcium, and copper and molybdenum.[6] Several of these interactions have been shown to occur in mammalian cells in culture.[7]

The results reported here are part of a study designed to assess trace-element interactions in the rabbit alveolar macrophage that is used as a cytotoxicity screening system. The parameters measured in these experiments were percent viability, viability index, and adenosine triphosphate (ATP) content, which M. D. Waters et al. (unpublished data) have shown to correlate highly with phagocytic activity following exposure to soluble metallic chlorides.

METHODS

The techniques used for the procurement and maintenance of rabbit alveolar macrophages are modified from the procedures described previously.[2] New Zealand white rabbits were killed by injection of sodium pentobarbital (150 mg) into the marginal ear vein. After a tracheostomy was performed, lung lavage in situ was carried out by instilling 30 ml of sterile saline in the lungs of each rabbit. The initial instillation was allowed to remain for 15 min. Five subsequent infusions of 30 ml each were withdrawn immediately. Lavage fluid that contained blood or mucous was discarded. The cellular yield consisted of an average of 30 to 40 million cells per rabbit; of these, 95 to 98% were macrophages, 1 to 3% were polymorphonuclear leukocytes, and 1 to 2% were lymphocytes.

The pooled cells were collected by centrifugation at 365 x *g* for 15 min at 5°C and were resuspended in 37°C medium 199 in Hanks' salt solution supplemented with 20% heat-inactivated fetal-calf serum, 100 units/ml penicillin, and 100 μg/ml kanamycin and streptomycin. The cell suspension was adjusted to 10^6 cells/ml, and

2 ml was added to 25-cm^2 tissue-culture flasks. The macrophages were allowed to attach for 3 hr at 37°C in a humidified atmosphere of 4% CO_2 and 96% air. At the end of this period, 2 ml of 2x concentrated test medium containing the dissolved metals was added to each flask and incubated for 20 hr. At the end of the 20-hr incubation period, the medium from each flask was poured off and retained. The attached cells were removed by trypsinization and added to the medium retained above.

Initial experiments (Table 1) were conducted as described previously[8] with only the cells that attached after 3 hr. The concentration responses were very similar when both attached and unattached cells were exposed as described above and all subsequent interaction experiments were performed as described here.

Simultaneous determinations of cell number per milliliter and cell viability by the trypan-blue dye exclusion technique were performed on a cell suspension using a Cytograf model 6301 (Biophysics Systems). Viability was expressed directly as a percentage of 1000 cells classified. The viability index was determined by multiplying (percent viability) x (number of cells in the experimental culture/number of cells in the control). The viability index is a parameter that defines the combined effect of a test compound on viability and cell number. Adenosine triphosphate was determined

TABLE 1

EC50 VALUES* FOR PERCENT VIABILITY AND VIABILITY INDEX† FOR METALLIC SULFATES IN RABBIT ALVEOLAR MACROPHAGES AFTER 20 HR

Compound	Percent viability (EC50), m*M*	Viability index (EC50), m*M*
$HgSO_4$	0.053 (0.040–0.071)	0.037 (0.021–0.051)
$CdSO_4$	0.079 (0.063–0.102)	0.051 (0.040–0.065)
VSO_4	0.171 (0.109–273)	0.048 (0.025–0.076)
$CuSO_4$	0.605 (0.526–0.702)	0.553 (0.469–0.647)
$Zn(NH_4)_2SO_4 \cdot 6H_2O$	0.673 (0.601–757)	0.493 (0.432–0.562)
$ZnSO_4$	0.812 (0.656–1.05)	0.519 (0.434–0.626)
$MnSO_4$	4.31 (2.54–9.94)	1.28 (0.868–2.20)
$NiSO_4$	5.91 (4.32–9.58)	3.00 (2.35–4.26)

*Effective concentration resulting in 50% response after 20 hr. 95% confidence intervals are shown in parentheses.

†Viability index = (percent viability) x (total intact cells in experimental culture/total intact cells in controls).

with a Du Pont model 760 Luminescence Biometer as described previously.[8] Adenosine triphosphate levels were adjusted per 10^6 cells and expressed as a percent of control.

The metals were prepared as 100m*M* stock solutions in deionized water, filtered through a 0.22-μm membrane, and stored frozen. Aliquots of these stocks were diluted in culture medium, minus serum, just prior to testing, and the pH was adjusted if necessary to between 6.2 and 7.4. Aliquots of each concentration of metal tested were frozen for atomic absorption spectrophotometry. Analysis of actual metal concentrations in the test media were determined on a Perkin—Elmer model 306 atomic absorption spectrophotometer equipped with a model HGA-2100 graphite furnace. Conditions used for atomic absorption spectrophotometry were those described in the Perkin—Elmer instrument manual.

A complete concentration-response curve was determined for each metal sulfate using a minimum of six concentrations run in triplicate to obtain the values reported. Each metal was tested with a minimum of four different pools of alveolar macrophages on different days. Experiments on trace-element interactions were performed at least twice, and each treatment concentration was examined in triplicate with the exception of the ATP levels, which were run in duplicate.

RESULTS AND DISCUSSION

The relative toxicity of a series of metallic sulfates tested in the rabbit alveolar macrophage over a 20-hr exposure period in vitro are shown in Table 1. The three most toxic metals of those tested are mercury, cadmium, and vanadium. Copper and zinc exhibited moderate toxicity, and the least toxic metals of those tested were manganese and nickel. The concentrations of these metallic sulfates, which caused a 50% decrease in cell viability and viability index (EC50 values), are similar to those published previously for the metallic chlorides.[1,2] When the same pool of macrophages was used in concentration-response experiments with $NiCl_2$ and $NiSO_4$, no significant differences were seen in the concentration response for percent viability, as shown in Fig. 1. In analogous experiments with zinc and cadmium, chlorides and sulfates exhibited the same concentration response with respect to viability and viability index.

Trace-element interactions that are most likely to affect the net toxicity of a crude sample are those which involve either metals in particularly high concentration in the sample or metals that are the more toxic. Of the more toxic metals (cadmium, mercury, and

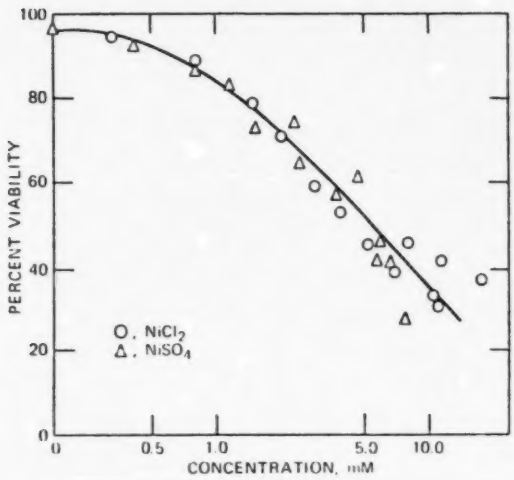

Fig. 1 Comparison of the effect of $NiCl_2$ and $NiSO_4$ on the percent viability of rabbit alveolar macrophages after 20 hr.

vanadium) examined with the alveolar macrophage, a number of interactions involving cadmium (e.g., with zinc, copper, and selenium) and mercury (with selenium) have been reported in whole animals.[9] A recent review of vanadium toxicology[9] suggests that vanadium may interact with copper.

The antagonistic interaction between cadmium and zinc is well-known. Initial studies with chicks[10] and later with mice and rats[11] showed that cadmium interfered with the metabolism of zinc, copper, and iron and that the toxic effects of cadmium could be largely overcome by administering additional zinc or copper. Exposure of rabbit alveolar macrophages to $CdSO_4$ (0.08m*M*), plus increasing concentrations of $ZnSO_4$ (from 0.08 to 0.16m*M*), showed increasing protection by zinc against cadmium toxicity, as shown in Fig. 2. All three parameters measured (percent viability, viability index, and ATP concentrations) increased up to 2-, 3-, and 4.4-fold, respectively, with increasing concentrations of $ZnSO_4$ up to 0.16m*M*. The lack of increased protection above 0.16m*M* $ZnSO_4$ is probably due to increasing toxicity of zinc, as indicated by the control cultures that contained $ZnSO_4$ alone.

Selenium in the form of selenite was first shown by Parizek et al.[12] to protect against the toxicity of both cadmium and mercury in whole animals. The effect of sodium selenite on the cytotoxic

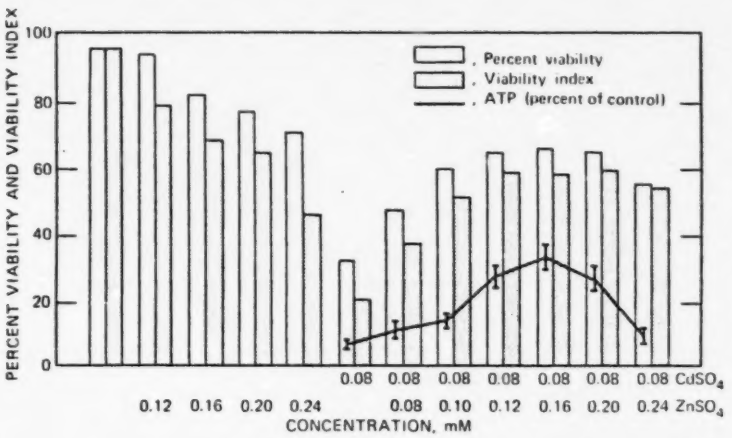

Fig. 2 Interaction of $CdSO_4$ and $ZnSO_4$ in rabbit alveolar macrophages after 20 hr. The standard errors per data point for percent viability and for viability index were less than 5% of the respective means. The standard errors per data point for ATP content are shown by vertical bars.

response observed in the alveolar macrophage due to cadmium and mercury salts is shown in Fig. 3 and 4. As little as 0.004m*M* of selenium in the presence of 0.08m*M* of cadmium or mercury (1 : 20 molar ratio) resulted in some protective effect on the alveolar macrophage in vitro. The protection observed in the cadmium—selenium experiment with 0.08m*M* of $CdCl_2$ and 0.016 to 0.032m*M* of Na_2SeO_3 resulted in increased percent viability, viability index, and ATP concentrations of 1.8-, 2.0-, and 2.1-fold, respectively. Parizek et al.[12] observed the protection of selenium against cadmium toxicity in the rat in the same range of selenium—cadmium molar ratios as observed in the macrophage.

The interaction between Na_2SeO_3 and $HgSO_4$ (Fig. 4) in the alveolar macrophage differs in two respects from that discussed above for selenium and cadmium. First, the protection is observed over a wider concentration range, with very little decrease in protection at the highest concentrations of selenium used. Second, the selenium protected to a greater extent against mercury toxicity, increasing all three parameters measured to nearly 100% of control values. This extensive protection by such low concentrations of selenium is particularly important since mercury is the most toxic element we have evaluated in this cytotoxicity screening system.

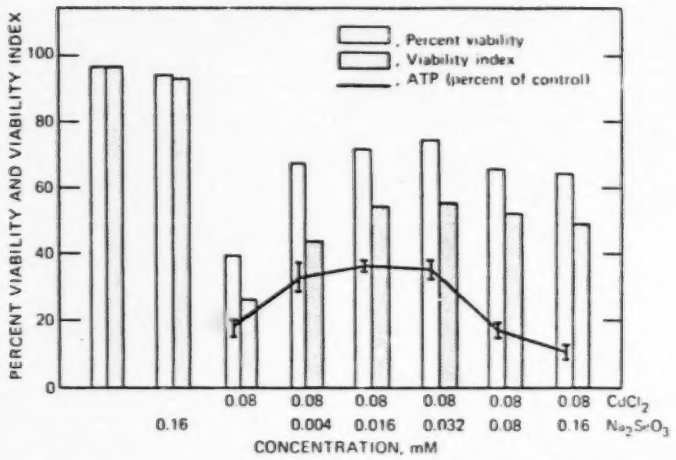

Fig. 3 Interaction of $CdCl_2$ and Na_2SeO_3 in rabbit alveolar macrophages after 20 hr. The standard errors per data point for percent viability and for viability index were less than 8% of the respective means. The standard errors per data point for ATP content are shown by vertical bars.

Both VSO_4 and NH_4VO_3 are highly toxic to alveolar macrophages in culture, ranking after mercury and cadmium in terms of percent viability. These two vanadium compounds and the vanadium oxides discussed in an earlier report[13] are the most cytolytic of the metals examined thus far. At the lowest concentrations of vanadium, where there was little effect on cellular viability, cell numbers were significantly decreased. As shown in Figs. 5 and 6, 0.1m*M* vanadium, which reduced the percent viability to 65 to 68%, decreased the viability index to 12%. The addition of increasing concentrations of either $CuCl_2$ or $CuSO_4$ resulted in increases in both the percent viability and the viability index. The increase in the viability index, however, was nearly four times the increase observed in the percent viability, indicating that copper protected against the lytic effect of vanadium. With the exception of the indirect evidence reviewed by Waters[9] which suggests that vanadium may interfere with copper metabolism, we know of no other experimental studies that have reported the protection of copper against vanadium toxicity.

When alveolar macrophages were exposed to increasing concentrations of divalent metal ions, the ATP content and phagocytic index of the macrophage decreased to a greater extent than viability

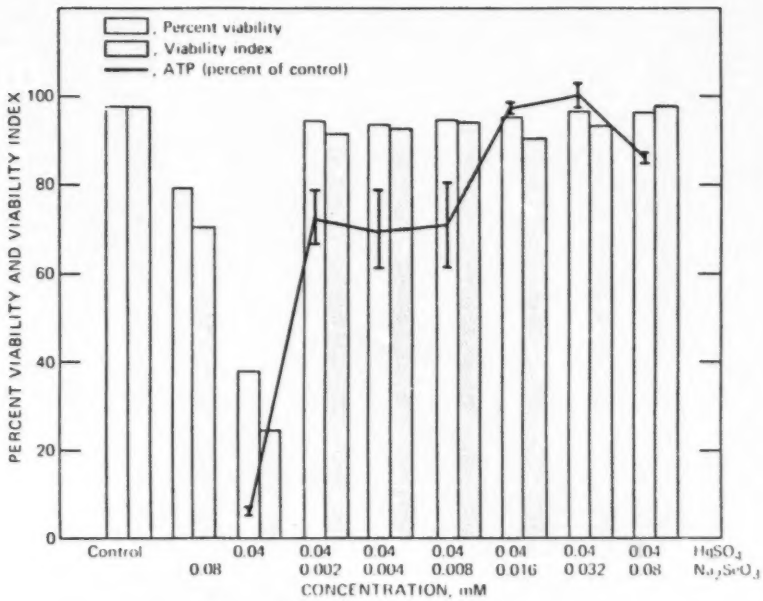

Fig. 4 Interaction of $HgSO_4$ and Na_2SeO_3 in rabbit alveolar macrophages after 20 hr. The standard errors per data point for percent viability and for viability index were less than 8% of the respective means. The standard errors per data point for ATP content are shown by vertical bars.

(M. D. Waters, personal communications). In the experiments reported here, the ATP content per 10^6 macrophages did initially increase with increasing viability as the concentration of antagonizing element increased. At the higher concentrations of the antagonizing element in every case except mercury and selenium, the ATP content decreased after reaching a maximum, but the percent viability remained higher. In these studies the ATP content of the antagonizing element alone was not measured, and ATP values per 10^6 macrophages are expressed as a percent of the control cultures. More extensive studies of the effect of these trace elements alone and in combination on the ATP content of the macrophage are now in progress, and the ATP values shown should be considered as relative values.

The supernatant fraction from a copper smelter particulate sample that was previously found to be highly toxic in the alveolar

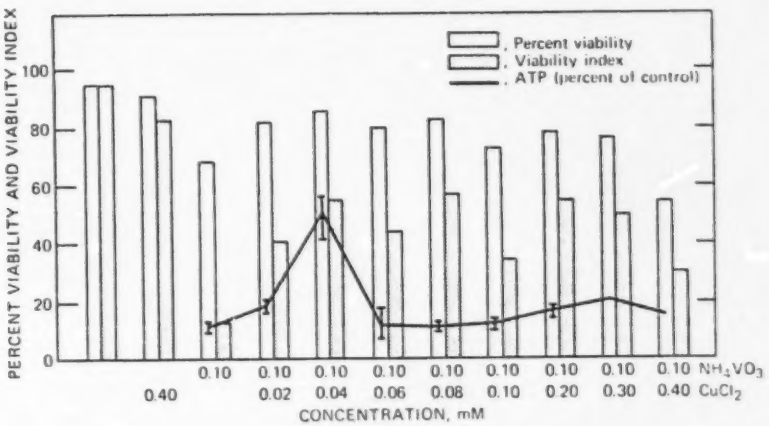

Fig. 5 Interaction of $CuCl_2$ and NH_4VO_3 in rabbit alveolar macrophages after 20 hr. The standard errors per data point for percent viability and for viability index were less than 10% of the respective means. The standard errors per data point for ATP content are shown by vertical bars.

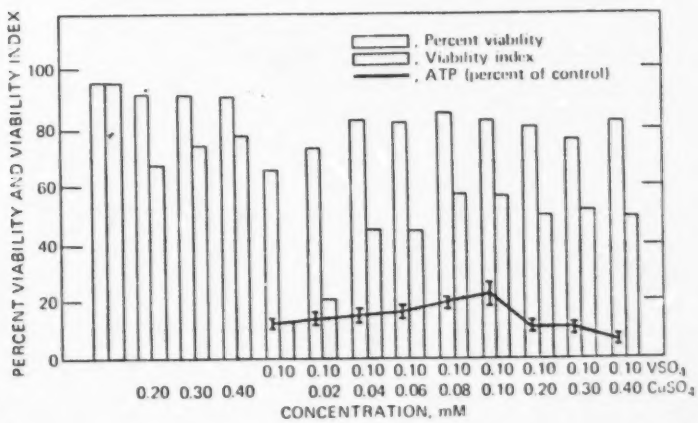

Fig. 6 Interaction of $CuSO_4$ and VSO_4 in rabbit alveolar macrophages after 20 hr. The standard errors per data point for percent viability were less than 5% and for viability index were less than 15% of the respective means. The standard errors per data point for ATP content are shown by vertical bars.

macrophage test system (EC50 for viability = 5 μg/ml)[3] had a predicted toxicity based on the elemental composition of nearly twice the actual toxicity.[4] The soluble supernatant fraction of the sample exhibited as much toxicity as the particulate itself. The sample contained the following elements: over 1% copper, arsenic, lead, antimony, and bismuth; and over 0.1% zinc, tin, selenium, silver, and cadmium. Elements in this sample which are known to interact antagonistically in whole animals include zinc and copper, selenium and arsenic, zinc and cadmium, and selenium and cadmium.

The supernatant fraction of this sample was incubated with alveolar macrophages alone and in the presence of nontoxic concentrations of cadmium, mercury, copper, zinc, and selenium (Fig. 7). In 0.15m*M* $ZnSO_4$, the percent viability and the viability index of the copper smelter sample increased 1.7- and 2-fold, respectively. Zinc is known to protect whole animals from both copper and cadmium toxicity, two elements that are present in this industrial particulate.

SUMMARY

In the evaluation of the rabbit alveolar macrophage as an in vitro test system for screening air and other crude particulate matter for

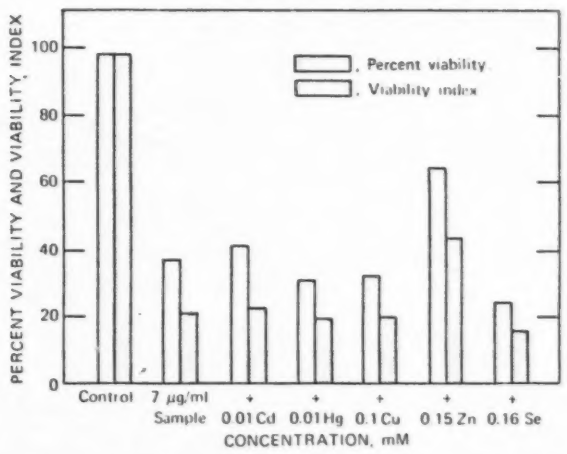

Fig. 7 Effect of cadmium, mercury, copper, zinc, and selenium on the toxicity of the supernatant fraction from a copper smelter sample (3 to 10 μm). The standard errors per data point for percent viability and for viability index were less than 10% of the respective means.

relative cytotoxicity, it is important that the alveolar macrophages in culture respond to trace-element interactions approximately as they would occur in a whole-animal or human-exposure situation.

Alveolar macrophages were shown to exhibit several trace-element interactions previously reported in whole animals. Zinc protected against cadmium toxicity in the macrophage, and selenium protected against both cadmium and mercury toxicity in a relative range previously reported for whole animals.

The alveolar macrophage may also provide a useful tool for screening metals and other elements for interactions, as suggested by the observation that copper protects the macrophages from the lytic effect of vanadium.

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